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SYNERGISMS IN PLANT DEFENSES AGAINST HERBIVORES: INTERACTIONS OF CHEMISTRY, CALCIFICATION, AND PLANT QUALITY[1]

MARK E. HAY AND QUAKER E. KAPPEL
University of North Carolina at Chapel Hill, Institute of Marine Sciences, Morehead City, North Carolina 28557 USA

WILLIAM FENICAL
University of California at San Diego, Scripps Institution of Oceanography, La Jolla, California 92093-0236 USA

Abstract. Many tropical seaweeds and benthic invertebrates produce both secondary metabolites and calcium carbonate ($CaCO_3$) particles or spicules that serve as possible defenses against consumers. To evaluate the relative defensive value of $CaCO_3$, secondary metabolites, and the potential synergistic or additive effects of the two, we made artificial agar-based "seaweeds" in which we manipulated algal organic content, $CaCO_3$, and the secondary metabolites produced by the calcified green seaweeds *Rhipocephalus phoenix, Udotea cyathiformis,* and *Halimeda goreauii,* all of which are relatively resistant to herbivores. The effects of these manipulations on herbivore feeding were evaluated using three different types of herbivores, the sea urchin *Diadema antillarum,* the amphipod *Cymadusa filosa,* and a mixed-species group of small parrotfishes. Addition of finely powdered $CaCO_3$ as 69% of food dry mass had no effect on feeding by parrotfishes, deterred feeding by *Cymadusa,* and deterred *Diadema* when food organic content was low but not when it was higher. Although calcification of algal tissues has generally been considered a structural defense that hardens seaweed thalli and makes them more resistant to attack, the decreased feeding on $CaCO_3$-containing foods in our assays occurred without any measurable alteration of food toughness.

At natural concentrations, semipurified secondary metabolites from *Rhipocephalus* or *Udotea* deterred feeding by all three herbivores. In most assays, feeding was depressed more by the addition of metabolites from *Rhipocephalus* or *Udotea* than by the addition of $CaCO_3$ even though $CaCO_3$ was added at 1.3–2.2 times the natural concentration for these plants. In contrast, the major metabolite from *Halimeda goreauii,* when tested alone, did not affect feeding by any of the herbivores. In two of our nine assays, the synchronous combination of $CaCO_3$ and secondary metabolites acted synergistically and deterred feeding significantly more than the sum of the effects of each tested separately. Mechanisms producing these synergisms are unknown, but it is possible that calcification could also be acting as a chemical defense by altering gut pH in ways that increase the potency of the secondary metabolites. It is common for chemical, structural, morphological, and nutritional deterrents to co-occur in individual prey species. For some plant–herbivore interactions, the combined effects of these characteristics can be much more than the sum of their separate effects.

Key words: calcification; chemical defense; complex interactions; marine; plant–herbivore interactions; seaweeds.

INTRODUCTION

Although many marine organisms are defended against consumption by both calcification and secondary metabolites, manipulative investigations of defensive characteristics almost invariably study these traits in isolation. This prevents the detection of synergistic effects or the rigorous determination of the relative importance of each type of defense. Organisms such as seaweeds, sponges, ascidians, and soft corals commonly contain both secondary metabolites and potential structural defenses in the form of calcium carbonate ($CaCO_3$) granules, spicules, or sclerites. Extensive field and laboratory assays have shown that secondary metabolites from these organisms can function as strong feeding deterrents (Hay 1991, Hay and Steinberg 1992, Lindquist et al. 1992, Paul 1992). Manipulative experiments on the deterrent qualities of spicules in invertebrates or calcified thalli in seaweeds are less extensive. Spicules from a temperate gorgonian coral (Gerhart et al. 1988), tropical gorgonians (Harvell et al. 1988, Van Alstyne and Paul 1992), and several species of tropical soft corals (Van Alstyne et al. 1992) significantly deter fish feeding, while spicules from a tropical ascidian (Lindquist et al. 1992) and a tropical sea whip (V. J. Paul and K. L. Van Alstyne, *unpublished manuscript,* cited in Van Alstyne et al. 1992) do not.

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$CaCO_3$-containing seaweeds are often relatively low preference foods for herbivores, and algal calcification has commonly been interpreted as a defense against grazers (Littler et al. 1983, Hay 1984, Lewis 1985, Paul and Hay 1986, Steneck 1988) even though some herbivores readily consume calcified algal tissues (Steneck 1982, Hay 1984, Lewis 1985, Padilla 1989). However, there have been few direct experimental tests of the effect of $CaCO_3$ addition on feeding. When powdered calcite was added to a carrageenin-based food, feeding by the sea hare *Dolabella* was reduced significantly (Pennings and Paul 1992), as was feeding by some Pacific damselfishes and surgeonfishes (Schupp and Paul 1994). These previous investigations clearly show that both $CaCO_3$ and chemical characteristics can affect prey susceptibility to predation, but they do not address the question of why these two traits so commonly co-occur.

Among the 70 species of tropical seaweeds studied by Paul and Hay (1986), 85% of the calcified species contained secondary metabolites, compared with only 39% of the uncalcified species, yielding a significant co-occurrence of calcification and secondary metabolites. Although it is less well documented, calcification and secondary metabolites also co-occur in ascidians, sponges, cnidarians, and other seaweeds (Hay 1984, Kingsley 1984, Faulkner 1992 [see also Faulkner's six other reviews that are referenced in his paper], Lindquist et al. 1992, Van Alstyne and Paul 1992). The common co-occurrence of $CaCO_3$ and chemical defenses in tropical prey has been suggested to be adaptive because the high diversity of tropical consumers limits the effectiveness of any single defensive trait (Lubchenco and Gaines 1981, Hay 1984, Lewis 1985, Paul and Hay 1986). Additionally, we hypothesize that $CaCO_3$ and chemical defenses could act additively or synergistically. We can envision how calcified particles or spicules might abrade protective gut linings or alter gut pH in ways that might enhance the detrimental effects of bioactive secondary metabolites.

Synergisms among chemical defenses occur in terrestrial systems where the deterrent effects of a plant secondary metabolite can be increased tremendously because the plant also produces small quantities of a nondeterrent compound that inhibits an insect's ability to alter the deterrent compound (Berenbaum and Neal 1985). Synergisms between chemical and structural defenses (such as silification in grasses), however, are not known for terrestrial plants.

Among marine organisms, potential synergisms between $CaCO_3$ and chemical defenses could be widespread given the frequent co-occurrence of the two characteristics. However, there are only two previous studies where potential synergisms can be assessed; neither study documents a significant synergism, although one study is suggestive. Gerhart et al. (1988) found that spicules and organic extracts from the gorgonian *Leptogorgia virgulata* tended to be more deterrent when combined than when each was tested alone. However, the effects of spicules alone and spicules plus extracts did not differ significantly in these assays, so neither a synergistic nor an additive effect could be rigorously demonstrated. When Pennings and Paul (1992) tested the effects of $CaCO_3$ and seaweed metabolites on feeding by the sea hare *Dolabella,* they found no synergistic effects.

In this study, we focused on three widely distributed green seaweeds that are calcified and produce unusual terpenes (*Halimeda goreauii, Udotea cyathiformis,* and *Rhipocephalus phoenix*). In field transplant studies conducted on numerous reefs scattered throughout the Caribbean, these species and others in each of these genera consistently have been shown to be relatively low preference foods for reef fishes and sea urchins (Ogden 1976, Hay 1981, 1984, Littler et al. 1983, Lewis 1985, Paul and Hay 1986). To evaluate the relative importance of $CaCO_3$, chemical defense, and the two together, we tested the effects of calcite particles, semipurified secondary metabolites, and the two together on feeding by (1) the amphipod *Cymadusa filosa,* (2) the sea urchin *Diadema antillarum,* and (3) a mixed-species group of small parrotfishes. In addition to representing three distinct taxonomic groups of herbivores, these grazers span the range of common mouth parts and feeding mechanisms seen among reef herbivores and form a continuum of the escalating herbivory that has been seen in marine communities through time. Steneck (1983) noted that: (1) the small mandibles of amphipods are representative of very ancient marine herbivores, and do minimal damage to larger seaweeds; (2) the stronger and more damaging teeth of urchins evolved more recently; and (3) the very powerful fused teeth of parrotfishes represent the most recent escalation of herbivory, and do tremendous damage to seaweeds.

Methods

Study site and organisms

This study was conducted at the National Oceanographic and Atmospheric Administration (NOAA) National Undersea Research Center on Key Largo, Florida, USA. Because green seaweeds in the order Caulerpales are well investigated chemically (Paul and Fenical 1987), commonly produce both unusual secondary metabolites and calcified thalli, and have been shown in field transplant experiments to be relatively low preference foods for reef herbivores (Hay 1984, Paul and Hay 1986, Paul and Fenical 1987), we focused our efforts on three species from different genera within this group.

Halimeda goreauii occurs throughout the Caribbean at depths of 3–80 m (Norris and Bucher 1982, Littler et al. 1989). It is a low preference food for reef fishes (Hay 1984, Paul and Hay 1986) but is consumed by *Diadema antillarum* if moved onto shallow reefs where

this urchin is common (Morrison 1988). On reefs near our study site, *H. goreauii* appeared to be the most common seaweed on the reef below a depth of ≈15 m. Plants used in our study were collected from a depth of 20 m on the seaward edge of Conch Reef.

Rhipocephalus phoenix and *Udotea cyathiformis* were collected at 1–3 m deep from mangrove-lined channels at Tarpon Basin. Each of these species occurs widely throughout the Caribbean and is known to be a low preference food for herbivorous reef fishes (Hay 1984, Paul and Hay 1986). Although these species are often found in seagrass beds and mangrove channels, they also commonly occur on reefs where they grow on hard substrate or in small patches of sediment trapped in the reef framework (Norris and Bucher 1982, Littler et al. 1989). As an example, Norris and Bucher (1982) found *R. phoenix* in 7 of 16 reef sites and 3 of 10 sandy or sand and rubble sites around Carrie Bow Cay, Belize; *U. cyathiformis* occurred in 2 of the reef sites and none of the sandy sites.

Rhipocephalus phoenix produces sesquiterpene secondary metabolites (Sun and Fenical 1979), as does *Udotea cyathiformis* (Paul and Fenical 1986, 1987). *Halimeda goreauii* produces a diterpenoid tetraacetate (Paul 1985). Although these types of metabolites have been proposed to function as chemical defenses against reef herbivores (Sun and Fenical 1979, Norris and Fenical 1982, Hay 1984, Paul and Fenical 1986, 1987, Paul and Hay 1986), these particular compounds have not been rigorously assayed in ecologically realistic experiments.

To evaluate the effects of these compounds and of calcium carbonate ($CaCO_3$) on feeding by herbivores, we ran feeding choice assays in the laboratory using three very different types of herbivores. These included: (1) small individuals of the sea urchin *Diadema antillarum* collected from shallow areas of coral rubble (test diameters ranged from ≈2–4 cm), (2) plant-dwelling amphipods (>95% were *Cymadusa filosa*) collected from calcareous seaweeds in the genera *Rhipocephalus, Udotea,* and *Penicillus* growing in a mangrove-lined channel at Tarpon Basin, Florida, and (3) a mixed-species group of small (6–8 cm) grassbed and reef parrotfishes (≈50% *Sparisoma radians* with the remainder made up of *Sparisoma aurofrenatum, S. viride, Scarus coeruleus, S. taeniopterus,* and *S. croicensis*) collected by seining a grassbed–mangrove border near Rodriguez Key, Florida.

Prior to its Caribbean-wide die-off due to disease the sea urchin *Diadema antillarum* was ubiquitous on reefs, seagrass beds, mangrove roots, and sand habitats throughout the Caribbean (Bauer 1980, Lessios 1988). Numerous field investigations have shown that when *Diadema* are common, their grazing significantly affects macrophyte populations and communities on both shallow and deep portions of coral reefs (Carpenter 1986, de Ruyter von Steveninck and Breeman 1987, Hughes et al. 1987, Lessios 1988, Morrison 1988) and in soft-substrate habitats near reefs (Ogden et al. 1973). *Diadema,* in conjunction with other urchin species, can also affect seaweed communities in mangrove channels (Taylor et al. 1986). Field investigations have shown that *Diadema* will eat calcified and chemically rich seaweeds like *Halimeda* and *Penicillus* (Ogden 1976), and that *Halimeda goreauii* and other unspecified *Halimeda* species increased in abundance on deeper portions of Caribbean reefs following the die-off of *Diadema* in 1983 (Hughes et al. 1987, Morrison 1988).

We chose to use a mixed-species group of parrotfishes in our assays because parrotfishes have been repeatedly shown to have a large impact on the structure of seaweed communities on coral reefs (Lewis 1985, 1986, Hay 1991). Because these impacts have been demonstrated in field experiments where grazing was due to a mixture of species and because little is known about the effects of feeding by specific species, we chose not to use one species and assume it was representative of parrotfish in general. We reasoned that using a mixed-species group might increase our among-replicate variance and thus diminish the statistical power of our tests but that if we found significant treatment effects, we hoped that these results might prove to be robust. Additionally, because two of our three algal species grow on both reefs and seagrass beds, we used a mixture of reef and grassbed parrotfish. Approximately 50% of the parrotfishes used in our assays were the grassbed species *Sparisoma radians*; the remainder of the fishes were juveniles of species usually found on reefs. Although *Sparisoma radians* feeds primarily from seagrasses, field observations show that it consistently includes calcified and chemically defended green algae (e.g., *Halimeda* and *Penicillus*) in its diet and that electivity coefficients for these species are equal to or higher than those for the seagrasses (Lobel and Ogden 1981). Targett et al. (1986) demonstrated that compounds from some *Halimeda* species deter feeding by this fish, but the potential effect of $CaCO_3$ was not assessed. This parrotfish would commonly encounter *Udotea* and *Rhipocephalus* in seagrass beds. The other parrotfish species are normally found on reefs where they could encounter all of the seaweed species we used.

We know little about the ecology or feeding preferences of the amphipod *Cymadusa filosa.* This amphipod occurred in very high numbers on green calcified seaweeds (*Udotea, Rhipocephalus,* and *Penicillus*) at one of our collection sites and so was included in our assays.

Making artificial foods

To test the effects on herbivore food choice of adding $CaCO_3$, secondary metabolites, and the two together, we modified methods used by Pennings and Paul (1992) so that we could make artificial algae with the desired characteristics. Our artificial seaweeds were made by mixing freeze-dried and finely powdered green algae (a

mix of *Enteromorpha, Cladophora,* and *Ulva,* all of which are commonly consumed by a variety of herbivores) into an agar base and forming this onto fiberglass window screening material that provided support and an internal uniform grid that allowed us to quantify amount eaten by counting the squares of the screen that had been cleared of algae (see Fig. 1). The artificial food was prepared by mixing 20 mL of water with 0.72 g of agar and heating this in a microwave oven until it boiled (≈40 s). The boiling water and agar were stirred and then poured into 16 mL of cold water containing 2 g of freeze-dried algae. This was stirred to assure uniform mixing. In one assay with *Halimeda* extract, we doubled the algal content of the food (i.e., 4 g) to see if the effects of $CaCO_3$ and secondary metabolites on feeding might be altered as a function of food reward per volume of "plant" consumed.

Calcified foods were made by mixing commercially available reagent-grade calcite (= $CaCO_3$) into the mixture as 69% of the food's total dry mass. This concentration approximates natural levels of $CaCO_3$ found in several species of *Halimeda* (50–85%), but is above the levels reported for species of *Rhipocephalus* (46–54%) and *Udotea* (32–47%) (Hillis-Colinvaux 1980). Because the additional calcite added volume as well as mass, we added more algae to these treatments so that the mass of algae per volume of our artificial food would stay constant across all treatments. Seaweeds deposit $CaCO_3$ as one of two crystalline structures, either calcite or aragonite (Borowitzka 1977). Although the seaweeds we studied contain aragonite, previous assays with other herbivores (Pennings and Paul 1992) and preliminary assays with our herbivores indicated that effects of $CaCO_3$ on feeding did not differ between incorporation of aragonite (obtained by digesting *Halimeda*) vs. calcite into the artificial foods. We used calcite because it is available commercially in reagent-grade quality.

When making artificial foods, we chose to make a uniform food type modeled roughly on *Halimeda opuntia,* one of the most abundant and widespread seaweeds in the Caribbean that is both calcified and chemically rich (Paul and Fenical 1986). Table 1 shows the organic content of our artificial foods compared with that of *Halimeda opuntia* and *H. goreauii.* Algae were blotted to remove surficial water prior to determining their volume by immersing them in a graduated cylinder of seawater.

Natural concentrations of secondary metabolites were added to our food by placing the freeze-dried algae (the mix of *Ulva, Enteromorpha,* and *Cladophora*) that was to be used in a small flask, adding the desired amount of metabolite (i.e., the amount isolated from an equivalent volume of the test alga) dissolved in diethyl ether, adding more ether until it covered all the algae, and then removing the ether with a rotary evaporator. This resulted in a uniform coating of metabolite on the algal particles prior to their being added into the agar. Control algae were treated identically but without addition of the metabolite.

TABLE 1. The organic content of our artificial foods compared to plants of *Halimeda opuntia* and *H. goreauii* (ash-free dry mass, means ± 1 SE).

Food type or plant species	Organic matter (mg/mL)	*n*
Uncalcified food with 2 g of algae	44 ± 0	5
Calcified food with 2 g of algae	45 ± 0	5
Uncalcified food with 4 g of algae	69 ± 0	5
Calcified food with 4 g of algae	67 ± 0	5
Halimeda opuntia	52 ± 3	10
Halimeda goreauii	75 ± 5	10

Semipurified secondary metabolites were obtained as follows. A known volume of fresh alga was ground in a blender in 1:2 methanol : dichloromethane, solids were filtered, solvents were removed by rotary evaporation from the remaining liquid extract, and this extract was partitioned between water and dichloromethane. The secondary metabolites were obtained in varying purities by silica thick-layer chromatography of the dichloromethane-soluble material. A portion of the semipurified metabolites was added at natural volumetric concentrations to the foods used in our assays. The larger portion was returned to Scripps Institution of Oceanography where the identities of the secondary metabolites were determined by nuclear magnetic resonance spectroscopy (NMR) and other spectral methods, and where the purities of the secondary metabolites were determined by high pressure liquid chromatography (HPLC) methods.

Foods were made by pouring the algal–agar mixture into the mold shown in Fig. 1. A razor blade was used to smooth the food into the mold and assure a uniform thickness. The standard mold was made from a rectangular piece of 2 mm thick Formica (a plastic laminate) with two 1.3 × 55.5 cm openings cut into it. A portion of window screen (1.6 × 1.8 mm openings) was clamped between the mold and a smooth sheet of polyvinyl chloride (PVC) plastic. Waxed paper beneath the screen prevented the food from sticking to the PVC sheet (Fig. 1). In each assay, a control food (i.e., without secondary metabolites or $CaCO_3$) was poured into one opening of the mold and the treatment food into the other. As the agar cooled, it took the shape of the mold and became firmly attached to the screen. The screens were then cut perpendicular to the food material so that a length of screen would contain rectangles of both treatment and control foods. Urchin assays used food that measured 7 × 14 squares (i.e., openings in the screen); fish assays used food measuring 7 × 7 squares.

Because amphipods eat much less per individual than do fishes or urchins, we needed food made with less total algal mass in order to have the amount eaten be sufficient to be measurable. This was achieved by omitting the mold and pouring the warm agar-based food

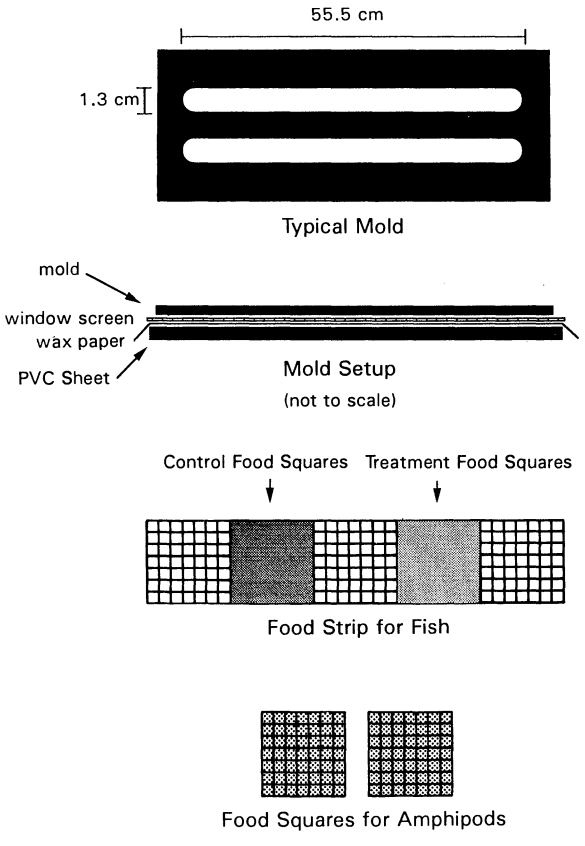

FIG. 1. The mold used to form our artificial algae onto screens and the resulting foods that were used in the assays.

directly onto a screen that was on waxed paper. An additional piece of waxed paper was placed on top of the hot food and screen, and a smooth block of PVC was used to press the food out to a uniform thickness. After cooling, the food would be stuck to the screen in a thin uniform layer that was cut into 7 × 7 square sections and offered to amphipods.

Because our calcified foods contained calcite as fine particles, they matched the chemical presence of $CaCO_3$ but not the increased toughness and hardness that characterizes many seaweeds that have their calcified particles arranged in an organized structural matrix. We attempted to measure differences in the force required to pierce our calcified and uncalcified foods using a penetrometer (Littler and Littler 1980) that had worked well on very soft seaweeds such as *Dictyota* and *Calonitophyllum* (Duffy and Hay 1991). Both food types were below the measurement potential of this device, indicating that differences in physical resistance to biting between our different agar-based foods would be unlikely to affect herbivore preferences.

Feeding assays

Assays with amphipods were run in 50 mL cups, each of which contained three amphipods and a simultaneous choice of treatment or control food. For urchin assays, two small urchins were placed in 2-L plastic bowls with small holes in the side that allowed a continual exchange of water when these were placed in a flow-through seawater system. Parrotfish assays used a similar design except mesh baskets were substituted for the plastic bowls and there were 2–4 fish per basket. In each assay, there were 20 separate containers holding each species of herbivore. These were all monitored periodically and algal foods were removed whenever (1) it appeared that one-half or more of either the treatment or the control food had been eaten, or (2) at the termination of the experiment (for replicates with low rates of feeding). If the herbivores in a replicate did not feed at all or ate all of both foods between our monitoring intervals, this replicate produced no information on relative palatability of the two foods and was excluded from consideration. The urchins and amphipods fed well in these assays and produced sample sizes that were always between 14 and 20. Because the fish seemed skittish and fed slowly, there were often replicates in which no feeding occurred; this resulted in usable sample sizes of only 9–14 for the fish assays. This also prevented us from doing as many assays with the fish as we did with the urchins and amphipods.

To see if the algal metabolites we added to the artificial foods degraded during our assays, uneaten portions of the treatment foods were extracted with 1:2 methanol : dichloromethane at the end of our feeding trials. This organic extract was then compared by thin layer chromatography to the initial compounds we had added to the food. Compounds were always still present and no breakdown products were detected. This method documents presence of the compounds but not their concentration. However, because these algal metabolites are not water soluble, there should be little, if any, loss to the water column (see Hay and Fenical 1988).

In all assays, amount of food eaten was determined by counting the number of screen squares from which the algal material had been completely removed (i.e., our "algae" come with the equivalent of graph paper embedded in them, so determining the area of alga removed is easy). In the absence of grazers, there is no change in the number of squares covered by algae, thus eliminating the need to run "controls" for changes unrelated to consumption (see Peterson and Renaud 1989).

Statistical analyses

In all of our assays, each individual herbivore had access to both a treatment and control food. By periodically monitoring the assays and pulling out individual replicates whenever half or more of either food had been consumed, we reduced the variance among replicates that would have been due to differences in herbivore hunger or willingness to feed in the test situations we established. Because a few individuals ate little during an entire assay or ate nothing for several

hours and then ate most of both foods before we were able to remove them, our efforts to minimize the "noise" of among-replicate variance in feeding rate that was unrelated to preference were only partially successful. However, by attempting to standardize amount eaten (i.e., about half of at least one of the foods) and minimize among-replicate variance in total food eaten, we achieved relatively powerful tests of how each treatment affected herbivore feeding preference.

The differences generated by each assay were tested for normality using SAS univariate procedures. Analyses of normally distributed data employed paired-sample t tests; non-normally distributed data were analyzed by the Wilcoxon signed-ranks test.

Because the paired assays generated one number for each replicate (i.e., the difference between control and treatment in amount eaten), we were also able to use ANOVA-type procedures to analyze for among-treatment differences in the degree to which each treatment (i.e., $CaCO_3$ alone, secondary metabolites alone, or the two together) affected feeding by each herbivore. If data were normally distributed and Cochran's test did not indicate unequal variances, we evaluated our findings using ANOVA followed by a Tukey test at $\alpha = 0.05$. Some analyses were conducted using nonparametric procedures (Kruskal-Wallis test followed by a nonparametric parallel of the Student-Newman-Keuls (SNK) as proposed by Dunn, see Zar [1984:200] at $\alpha = 0.05$) when: (1) data sets were not normally distributed, (2) Cochran's test indicated unequal variances among treatments, and (3) attempts to remedy this via arcsine (and other) transformations failed. This statistical approach provides a rigorous way to evaluate among-treatment differences in effects on feeding; however, because grazing in replicates within each treatment was often stopped after different periods of time (e.g., some replicates ate rapidly and were stopped quickly while others ate very little during the entire assay period) there was considerable among-replicate variance that resulted from differences in feeding rate rather than feeding preference. This increased variance may have caused these multiple-contrast procedures to have less power than the paired-sample procedures described earlier.

In three assays, the deterrent effect of $CaCO_3$ and secondary metabolites combined appeared much greater than the deterrent effect of either in isolation. To see if this increased deterrence was additive or synergistic, we randomly paired control minus treatment differences from tests with $CaCO_3$ alone with differences from tests of secondary metabolites alone (i.e., to get the mean difference and variance that would be expected from the summation of the separate effects of $CaCO_3$ alone and secondary metabolites alone), summed these differences, and tested the mean of these summed differences (using a t test or Mann-Whitney test depending on normality of the data) against the mean from the assay where both $CaCO_3$ and secondary metabolites were synchronously applied to the food. If the degree of feeding deterrence seen when $CaCO_3$ and secondary metabolites were added synchronously was significantly greater than the additive effects of each tested alone, then we interpreted this as indicating a synergistic effect. No significant difference would indicate that the effect had been additive rather than synergistic.

Results

The effects of (1) calcite (as 69% of food dry mass), (2) partially purified seaweed secondary metabolites (at natural concentrations), and (3) both calcite and secondary metabolites together on feeding by the amphipod *Cymadusa filosa,* a mixed-species group of parrotfishes, and the sea urchin *Diadema antillarum* are shown in Figs. 2–4.

For *Rhipocephalus,* ≈75% of the semipurified fraction used in our assays was composed of the linear sesquiterpene rhipocephalin; fats and traces of the minor metabolite rhipocephenal (see Sun and Fenical 1979 for the structure) made up the remainder of this fraction. Parrotfishes were not deterred by calcite added as 69% of food dry mass, but all other treatments (i.e., those containing rhipocephalin) significantly deterred feeding by all three herbivores (Fig. 2). Feeding by *Cymadusa* was reduced 46% by the addition of calcite alone; however, the algal compound alone or in combination with calcite decreased feeding by 92 and 97%, respectively. The latter two treatments did not differ in their deterrence, but each reduced feeding significantly more than the addition of calcite alone (Kruskal-Wallis test, $P < 0.001$, followed by a nonparametric parallel of the SNK at $\alpha < 0.05$). Feeding by the sea urchin *Diadema* was reduced significantly ($P < 0.001$, Fig. 2) and by similar amounts (68–88%, Kruskal-Wallis $P = 0.630$) by all three treatments. Effects on parrotfishes were more complex. Calcite alone had absolutely no effect, the rhipocephalin fraction alone diminished feeding by a significant 62%, but the combined effect of calcite and the rhipocephalin fraction was significantly more deterrent (reducing feeding by 95%) than the effect of either alone ($P = 0.002$, ANOVA, followed by Tukey's test at $\alpha < 0.05$). When the additive effects of calcite alone and secondary metabolite alone were calculated and compared with the reduction in feeding that resulted from the simultaneous combination of calcite and secondary metabolite, there was a clear synergistic effect that exceeded what would have been expected from an additive interaction ($P = 0.028$, t test). A portion of this synergism could have been generated by what appeared to be greater rates of feeding (especially on the control diet) in replicates testing the combined effects of both calcite and the secondary metabolite (see Fig. 2). However, an ANOVA comparing the total amounts eaten (i.e., control plus treatment squares eaten) among the three different treatments indicated that there were no significant dif-

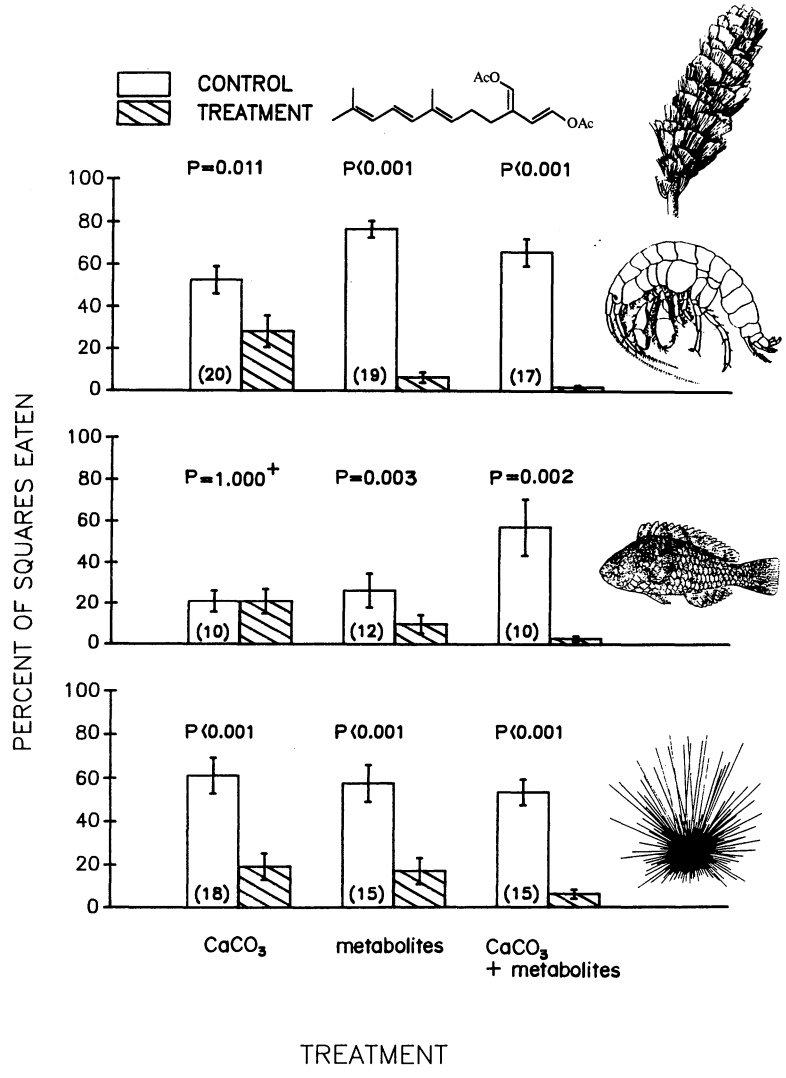

FIG. 2. The effects of adding calcite (as 69% of food dry mass), natural volumetric concentrations of the secondary compound rhipocephalin (structure diagrammed) from *Rhipocephalus phoenix,* and both calcite and rhipocephalin simultaneously on feeding by the amphipod *Cymadusa filosa,* a mixed-species group of parrotfishes, and the sea urchin *Diadema antillarum.* Histogram bars show means ± 1 SE. *P* values are from the paired-sample *t* test or Wilcoxon signed-ranks test (depending on normality of the data). All values are one-tailed except for those marked with a +. Numbers in parentheses at the base of the histogram bars are sample sizes. Data are presented in percentages to facilitate comparisons among herbivores. Statistical analyses were conducted using the numbers of squares completely eaten.

ferences in total feeding among the three treatments ($P = 0.334$). Thus, for *Rhipocephalus* (Fig. 2), (1) amphipods were deterred by either calcite alone or chemistry alone, with chemistry being the greater deterrent, and (2) parrotfish were unaffected by calcite alone but were deterred by chemical defenses alone. When calcite and secondary metabolites were added in combination, they acted synergistically and deterrence was increased significantly compared to the effects of chemistry alone or the additive effects of calcite alone plus secondary metabolite alone. (3) The sea urchin was deterred equally by calcite alone, secondary metabolite alone, or both together.

Separation of *Udotea* extract produced two fractions containing secondary metabolites. The bottom compound shown in Fig. 3 is the acyclic sesquiterpene flexilin (Paul and Fenical 1987). It comprised 36% of its fraction with fats and plasticizer (apparently from a faulty seal in our blender) making up the remainder. The top compound shown in Fig. 3 is a related sesquiterpene that does not have a common name. It comprised 61% of its fraction with fats making up the remainder. These compounds were reported previously from *Udotea cyathiformis, Penicillus capitatus,* and *Rhipocephalus phoenix* (Paul and Fenical 1986, 1987). Before the end of our feeding assays using amphipods and *Udotea* extracts, all of our amphipods died. This occurred in all replicates and treatments regardless of

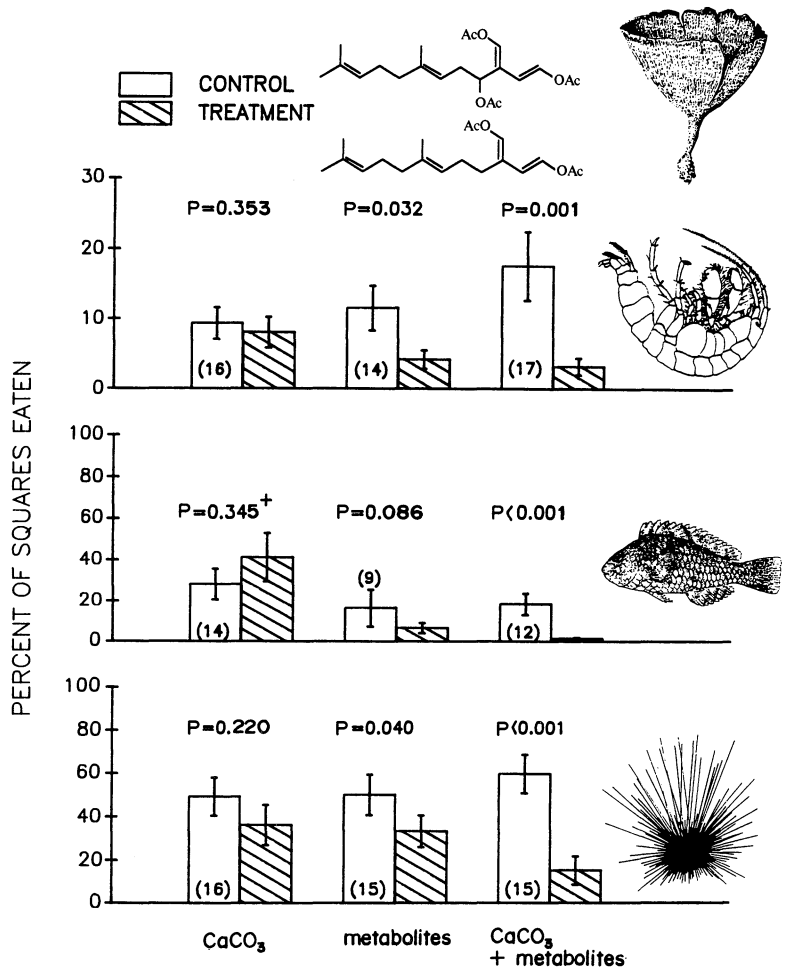

FIG. 3. Effects of calcite and secondary metabolites from *Udotea cyathiformis* on feeding by herbivores. The upper metabolite is a sesquiterpene that has no common name; the lower compound is the acyclic sesquiterpene flexilin. The inverted amphipod indicates that all amphipods, in all treatments, died during these assays (this may have been due to local spraying for mosquitoes). Note the different scales for percent eaten. Symbols and analyses are as in Fig. 2.

the presence or absence of *Udotea* metabolites, and it also occurred in our other containers that were holding amphipods not being used in the assays. Because crustaceans are very sensitive to many insecticides, we suspect that the die-off may have been due to local spraying for mosquitoes and not to any properties of *Udotea.*

Although amphipods in the *Udotea* assays ate some food before dying and this assay produced analyzable data, these data should be interpreted with caution because feeding rates were only 20–30% of levels seen in the other two assays with amphipods. As an example, calcite significantly depressed amphipod feeding in both the *Rhipocephalus* (Fig. 2) and *Halimeda* (Fig. 4) assays; the lack of a detectable effect in this assay may be due to the minimal total feeding that occurred. However, even with these minimal feeding rates, the simultaneous addition of the two fractions containing the *Udotea* compounds significantly decreased feeding (Fig. 3). As with *Rhipocephalus,* secondary metabolites from *Udotea* appeared more deterrent than calcite (Fig. 3); however, these differences were not significant when analyzed by the more rigorous but less powerful Kruskal-Wallis test ($P = 0.212$). In tests assaying the two *Udotea* fractions separately, the flexilin-containing fraction alone diminished amphipod feeding by a significant 96% ($P = 0.001$, paired t test, $n = 9$), and the fraction containing the related compound decreased feeding by a significant 99% ($P = 0.0008$, $n = 14$).

Feeding by parrotfishes was decreased significantly by the simultaneous addition of calcite and the fractions containing *Udotea* secondary metabolites, but

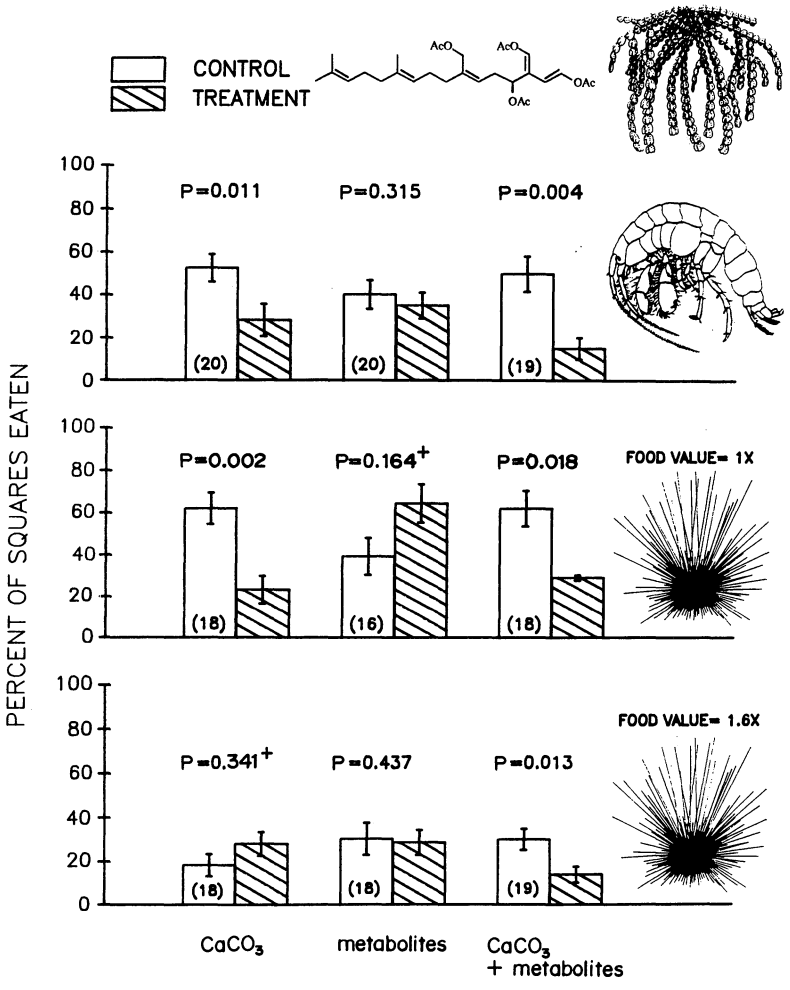

FIG. 4. Effects of calcite and a secondary metabolite from *Halimeda goreauii* on feeding by herbivores. Comparisons of the lower two graphs show how effects on feeding may be altered by changes in organic content of the food. The lower graph shows relationships when organic content of the food is similar to that of *Halimeda goreauii*. In all other assays, the organic content of the food is more similar to that found in *H. opuntia* (see Table 1). Symbols and analyses are as in Fig. 2.

their feeding was not significantly reduced by either calcite alone or the metabolite-containing fractions alone (Fig. 3). However, fishes fed very little in either of the *Udotea* treatments where they had access to metabolite-containing foods. Total feeding in the two assays where some of the food contained secondary metabolites was significantly lower than in the calcite alone assay ($P \leq 0.001$, ANOVA followed by Tukey's test at $\alpha < 0.05$). The mean ($\pm$ 1 SE) total number of squares consumed (control + treatment) in the three assays was as follows: calcite alone = 34 $\pm$ 8 (= 35% of available food); secondary metabolites alone = 6 $\pm$ 3 (= 6%); calcite and secondary metabolites = 7 $\pm$ 2 (= 7%).

Other workers have seen this pattern of reduced feeding on both treatment and control foods when fishes are given foods containing compounds that make them regurgitate but that they are unable to taste (N. Lindquist, *personal communication*). We interpret these results as an indication that the *Udotea* metabolites, either with or without $CaCO_3$ present, depress parrotfish feeding on both treatment and control foods. So few fish fed in the assay with compounds alone that we suspect our inability to show a significant treatment vs. control contrast is more a problem of statistical power and fish behavior than of any lack of effect. Because of these problems, we are reluctant to interpret these data (Fig. 3) as indicative of an important synergism between compounds and $CaCO_3$ in this particular test.

All treatments in the *Udotea* assays tended to diminish urchin feeding, but the decrease was significant

only for those assays with the metabolite-containing fractions, either alone or with $CaCO_3$ (Fig. 3). $CaCO_3$ diminished feeding by a nonsignificant 26% ($P = 0.220$, Wilcoxon paired-sample test, $n = 16$), the fractions containing the two secondary metabolites decreased feeding by a marginally significant 34% ($P = 0.040$), and the combination of metabolites and calcite decreased feeding by a significant 74% ($P = 0.0001$, Fig. 3). In this case, the greater apparent effectiveness of secondary metabolites and calcite together was additive rather than synergistic. The additive effects of calcite alone plus secondary metabolites alone did not differ significantly from the effects seen when both calcite and secondary metabolites were synchronously included in the food ($P = 0.232$, t test). As with the amphipod experiments, separate assays with the flexilin-containing fraction alone and the related compound fraction alone produced significant feeding reductions of a very similar magnitude (48 and 46%; paired t test, $P = 0.007$, $n = 16$ and $P = 0.026$, $n = 14$, respectively).

The extract of *Halimeda goreauii* contained one major secondary metabolite. The unnamed diterpenoid tetraacetate shown in Fig. 4 made up 52% of the fraction used in our assays with sterols comprising the rest of the mixture. This compound was previously described from collections of this species made in the Bahamas and Florida Keys (Paul 1985). The deterrent compounds halimedatetraacetate and halimedatrial that are common components of most Caribbean species of *Halimeda* (Paul and Fenical 1986, Targett et al. 1986, Hay et al. 1988, Paul and Van Alstyne 1988) were not found in our collection of *Halimeda goreauii*.

Calcite was an effective deterrent against amphipods ($P = 0.011$, paired t test); however, against urchins, the effectiveness of calcite was dependent on the concentration of freeze-dried algae added to the food. When 2 g of algal material was mixed into our agar-based food, the calcite-treated food was deterrent ($P = 0.002$, Wilcoxon paired-sample test); when we increased the algal material to 4 g, adding calcite had absolutely no deterrent effect on feeding ($P = 0.341$, Wilcoxon paired-sample test, bottom of Fig. 4). When placed in food with higher algal content, the *Halimeda* metabolite and calcite acted synergistically to significantly diminish urchin feeding. When tested separately, neither calcite alone nor the metabolite alone had any effect on urchin feeding (Fig. 4, bottom). Additionally, the additive effects of calcite alone plus secondary metabolite alone were significantly less deterrent than the effects seen when they were synchronously available in a single food ($P = 0.040$, t test). For the food with lower algal content, depression of urchin feeding on the calcite-containing and chemically rich food could be explained by the effect of calcite alone (Fig. 4).

Because of time limitations at this field site, we were unable to do more than this one assay on the interactive effects of secondary metabolites, calcite, and algal concentration on herbivore food choice. We were, however, able to determine the volumetric organic concentration (ash-free dry mass) of our various foods and of the two most common *Halimeda* species on reefs near Key Largo, Florida. Our higher algal content food (ash-free dry mass 67–69 mg/mL) contained ≈92% of the organic content found in *Halimeda goreauii* and 133% of that found in *H. opuntia* (Table 1). Our lower algal content food (ash-free dry mass 44–45 mg/mL) was more like *Halimeda opuntia* (52 mg/mL). Thus, the artificial food in which we observed a synergistic effect between calcite and the *H. goreauii* metabolite was a food that approximated *H. goreauii* reasonably well in terms of organic content.

Discussion

In our assays testing *Rhipocephalus phoenix* characteristics against parrotfish and *Halimeda goreauii* characteristics against urchins (Figs. 2 middle and 4 bottom), adding both secondary metabolites and calcite worked synergistically to decrease feeding. In a third case (urchins vs. *Rhipocephalus*), the greater degree of feeding reduction on foods with both secondary metabolites and calcite was additive rather than synergistic (Fig. 3, bottom). In the other six of our nine assays, neither synergistic nor additive effects were obvious.

Algal concentration in the food (i.e., reward per bite) also interacted with plant defensive characteristics to affect herbivore food choice. When our agar-based food was made with 2 g of freeze-dried algae (organic content per volume mimicking *Halimeda opuntia*), *Diadema* feeding on the calcite-containing food decreased significantly in two of three assays and decreased by 26% in the nonsignificant assay (Figs. 2–4). When reward per bite was increased by adding 4 g (instead of 2 g) of algae to the agar (organic content per volume mimicking *H. goreauii*), calcite alone had absolutely no deterrent effect on *Diadema* feeding (Fig. 4). Another investigation of how changing food reward alters the effectiveness of chemical defenses shows a roughly similar pattern; secondary metabolites deterred reef fishes from feeding on low protein foods but became ineffective as protein levels were raised (Duffy and Paul 1992).

Previous studies noted that a large proportion of calcified tropical seaweeds were also chemically defended and speculated that these multiple types of defenses were advantageous in tropical environments because there was such a high diversity of herbivore types that any single deterrent characteristic was unlikely to be broadly effective (Hay 1984, Paul and Hay 1986). A similar argument could be advanced for tropical benthic invertebrates, many of which produce both defensive secondary metabolites and calcified or siliceous spicules (Harvell et al. 1988, Lindquist et al. 1992, Paul 1992, Van Alstyne and Paul 1992, Van Alstyne et al. 1992). The potential synergistic or additive effects of both chemical defenses and $CaCO_3$

have rarely been addressed, and the two studies that are available did not detect significant synergisms (Gerhart et al. 1988, Pennings and Paul 1992). Two of our nine assays detected synergisms between secondary metabolites and $CaCO_3$. With so few studies available on this topic, it is premature to try and assess the relative frequency of synergistic vs. additive vs. noninteractive effects of multiple defensive characteristics. Our data do, however, indicate that significant synergisms can occur and should be considered in investigations of prey defenses.

Calcification of seaweeds has generally been viewed as deterring herbivory by making seaweeds harder and more difficult to bite or by diminishing their nutritional value due to the addition of indigestible structuring materials (Littler and Littler 1980, Steneck 1983, 1986, Hay 1984, Duffy and Hay 1990, Targett and Targett 1990, Duffy and Paul 1992, Pennings and Paul 1992). Our methods of adding calcite to the artificial foods did not increase food toughness and did not change the nutritional value of the foods (i.e., algal concentration per bite or per volume was held constant); however, calcite alone significantly reduced feeding in most of our assays with amphipods and sea urchins (Figs. 2–4). Pennings and Paul (1992) show a similar effect in tests with the herbivorous sea hare *Dolabella,* and Schupp and Paul (1994) also show that $CaCO_3$ addition diminishes feeding by surgeonfishes and damselfishes.

These findings do not diminish the potential importance of calcification in increasing algal toughness and thus diminishing consumption (Littler and Littler 1980, Steneck and Watling 1982, Steneck 1983), but they do show that calcification can also affect feeding via some other mechanism.

Calcification as a chemical defense

One potential mechanism by which $CaCO_3$ might interfere with herbivore feeding is the buffering effect that calcium carbonate would have on the gut pH of the numerous herbivores that rely on acid-mediated digestion. Ingested calcite might (1) directly inhibit digestion by neutralizing acid in the guts of herbivores that rely on acidic guts to lyse plant cells (Horn 1989), (2) indirectly affect digestion by altering the effectiveness of digestive enzymes, many of which function well only within a narrow range of pH, or (3) increase the effects of co-occurring secondary metabolites if their activity is pH sensitive as appears to be the case with polyphenolics (Feeny 1970). Given calcium carbonate's ability to buffer acids, $CaCO_3$ might be expected to have its largest effects on consumers with acidic guts. This hypothesis is consistent with the observation that both *Diadema* (gut pH 5.0–6.5; Lewis 1964) and herbivorous amphipods related to the *Cymadusa* we studied here (e.g., *Marinogammarus* spp. with a gut pH of 5.2–6.4; Martin 1966) have acidic guts, and both *Diadema* and *Cymadusa* were deterred by calcite. In contrast, parrotfishes, which have alkaline guts (Horn 1989), were not deterred by the addition of calcite. Similar assays run with Pacific reef fishes also show that addition of $CaCO_3$ to artificial foods decreases feeding by fishes with acidic but not alkaline guts (Schupp and Paul 1994). Because parrotfishes do not have acidic guts, calcite-mediated change in gut pH is an unlikely explanation for the synergistic effect on parrotfish feeding that was noted when both calcite and rhipocephalin were present in the food (Fig. 2). Our understanding of digestive physiology and biochemistry in marine herbivores is so incomplete that we cannot confidently suggest plausible reasons for why we find synergisms between secondary metabolites and $CaCO_3$ in assays with some herbivores and compounds but not in others. Even for the best studied groups, like herbivorous fishes (see Horn 1989 for an excellent review), mechanisms of digestion are just beginning to be investigated.

Seaweed secondary metabolites similar to those we assayed are known to rapidly convert, by apparent enzymatic hydrolysis, from less active to more active forms when the plant is damaged (Paul and Van Alstyne 1992). This conversion could be facilitated or retarded by changes in gut pH.

Alternatively, small crystals of calcite could abrade gut linings and allow compounds to act in situations where they might normally be thwarted by a thick gut epithelium (Bernays et al. 1989, Tugwell and Branch 1992). It has been suggested that the spear-like points on mineralized crystals that accumulate in some terrestrial plants act to pierce gut linings and allow entrance of plant toxins (Saki et al. 1972). Synergisms between chemical and supposedly structural defenses have also been suggested for a gorgonian soft coral that produces both calcified spicules and defensive secondary metabolites (Gerhart et al. 1988). Given the common co-occurrence of $CaCO_3$ and chemical defenses in both seaweeds (Hay 1984, Paul and Hay 1986) and a wide variety of marine invertebrates (Van Alstyne and Paul 1992), these types of synergisms could be common and widespread both taxonomically and geographically. However, progress in our understanding of defensive synergisms among various characteristics of marine organisms is unlikely to advance significantly until we achieve a greater understanding of both the digestive physiology of consumers and the specific mode of action of defensive metabolites.

Did calcified seaweeds evolve in response to herbivory?

Calcium carbonate deposition occurs in >100 genera of marine, freshwater, and soil algae (Borowitzka 1977). Direct experimental tests show that $CaCO_3$, even in the absence of increased hardness, can decrease feeding by amphipods, urchins, gastropods, and some fishes (Figs. 2–4; Pennings and Paul 1992, Schupp and Paul 1994). Because virtually all of these herbivore groups

diversified and evolved more powerful plant feeding abilities during the late Cretaceous and early Cenozoic (Steneck 1983), one might expect to see calcified seaweeds emerge during this time period. Although this scenario of escalating herbivory during the late Cretaceous providing strong selection for $CaCO_3$ deposition in seaweeds seems well reasoned, the fossil record of calcified seaweeds is good and shows that calcified algae with a variety of anatomical characteristics that diminish herbivore damage occurred 100×10^6 years before herbivory escalated (Steneck 1983, 1992). Calcified green seaweeds like those used in this investigation first appeared $\approx 180 \times 10^6$ years ago in the mid-Jurassic (Hillis-Colinvaux 1980). This precedes by $> 100 \times 10^6$ years the evolution of reef fishes that could exploit seaweeds (Choat and Bellwood 1991) or the period when macrophyte grazing urchins became common (Steneck 1983). Calcified red algae are known from the Cambrian (Steneck 1983), thus preceding powerful fish and urchin grazers by as much as 500×10^6 years.

In many seaweeds, calcification occurs as photosynthesis raises the pH of water-filled spaces between algal cells and causes precipitation of $CaCO_3$ (Borowitzka 1977). Thus, calcification may have evolved as a simple consequence of certain algal anatomies and the chemistry of photosynthesis in seawater. Millions of years later, this preadaptation appears to have allowed certain calcified seaweeds to radiate and increase in abundance as herbivores removed more palatable or less resistant competitors from many habitats in tropical seas (Steneck 1983, 1986, 1992). Within this context it seems that the more interesting question becomes what has selected against calcification in most seaweeds rather than what has selected for it in the few (Borowitzka 1977).

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